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Thermodynamics of partitioning of substance P in isotropic bicelles

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The temperature dependence of the partition of a neuropeptide, substance P (SP), in isotropic ($q = 0.5$) bicelles was investigated by using pulsed field gradient NMR diffusion technique. The partition coefficient decreases as the temperature is increased from 295 to 325 K, indicating a favorable (negative) enthalpy change upon partitioning of the peptide. Thermodynamic analysis of the data shows that the partitioning of SP at 300 K is driven by the enthalpic term (ΔH) with the value of -4.03 kcal mol^{-1}, while it is opposed by the entropic term ($-T\Delta S$) by approximately 1.28 kcal mol^{-1} with a small negative change in heat capacity (ΔC_p). The enthalpy-driven process for the partition of SP in bicelles is the same as in dodecylphosphocholine (DPC) micelles, however, the negative entropy change in bicelles of flat bilayer surface is in sharp contrast with the positive entropy change in DPC micelles of highly curved surface, indicating that the curvature of the membrane surface might play a significant role in the partitioning of peptides. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: substance P; isotropic bicelle; PFG NMR; partitioning; partition coefficient; diffusion coefficient

Introduction

The interaction of peptides with biological membranes is central to a number of biological processes such as the insertion and folding of peptides in membranes, the membrane fusion through the membrane fusion peptides, and the membrane-mediated mechanism of peptide–receptor interaction. A large number of studies have been made on the interaction of peptides with model membranes by using a variety of techniques [1–4], and the NMR spectroscopy has been a major technique in such studies [5,6]. For high-resolution NMR, the model membranes have to reorient fast and isotropically in solution with a correlation time of nanosecond scale. Due to this limit, the micelles or small unilamellar vesicles (SUV) have been widely used as a model membrane in NMR measurements [4,7,8]. Recently, the bicelles that consist of long- and short-chain lipids have drawn much attention as a model membrane for NMR study of peptide–membrane interaction due to the flat surface of long-chain phospholipid bilayers with a discoidal shape [9–12]. In particular, the small isotropically tumbling bicelles ($q < 1$) reorient in solution fast enough to provide highly resolved NMR spectra for bicelle-bound peptides [13–15], and many conformational studies of peptides with isotropic bicelles have been reported [14,16–18]. However, the studies on the partitioning (or binding) of peptides to the bicelles are scarce to date [19,20], even though they could provide better insights into the nature of the interactions between the peptides and biological membranes.

The binding of peptides has been observed as depending on many factors such as the net charge and binding conformation of peptides, hydrophobic property of peptide side chains, surface charge density of membrane, types of membrane lipids, and aggregation types (or shapes) of lipids [6,21,22]. However, it is rather difficult to separate the effects of many different factors due to their cooperative nature. Isotropic bicelles appear to be a suitable membrane mimetic system to separate one of such factors, the membrane curvature, in binding of peptides on the membrane surface, since the surface of bicelles is flat in contrast to the curved surface of micelles, while the two mimetic systems are similar in overall size.

Substance P (SP) is a neuropeptide composed of 11 amino acids (Arg^1-Pro^2-Lys^3-Pro^4-Gln^5-Gln^6-Phe^7-Phe^8-Gly^9-Leu^{10}-Met^{11}-NH$_2$), which is thought to be involved in many important physiological processes including pain transmission, inflammation, blood flow, salivation, and various muscle contractions [23,24]. This wide range of physiological activities of SP has been ascribed to the lack of selectivity for a specific receptor type. Thus, SP is known to activate three membrane-embedded receptor subunits with more or less different extents of potencies [24]. In addition, the lipid membrane is expected to perform an important role in the biological activity of SP either by increasing the concentration of SP at the surface of membrane or by inducing and stabilizing the physiologically active conformation of SP. Indeed, many conformational studies of SP in various membrane mimetic systems revealed that the membrane induces well-defined secondary structures in the mid-region of SP [7,8,25,26].

In the present study, we have measured the temperature dependence of the partition coefficient of SP in isotropic ($q = 0.5$) bicelles using pulsed field gradient (PFG) NMR diffusion technique. Thermodynamic analysis of the data shows that the transfer of SP from water into lipid bilayers in bicelles is enthalpy-driven with a negative entropy change. The result is in sharp contrast with the partitioning of SP in the dodecylphosphocholine (DPC) micelles,

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where the entropic term as well as the enthalpic term makes a favorable contribution (positive entropy change) to the binding of SP [4,6,27], indicating that the curvature of the membrane surface might play a significant role in the partitioning of peptides.

Materials and Methods

Sample Preparation

SP was obtained from Sigma Chemical Co., and used without further purification. 1,2-dimyristoyl-*sn*-gylcero-3-phosphatidylcholine (DMPC) and 1,2-dihexanoyl-*sn*-gylcero-3-phosphatidylcholine (DHPC) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Isotropic bicelle solution was prepared by dissolving DMPC and DHPC in 0.5 ml of sodium acetate buffer solution (pH = 6.0, 0.1 M NaCl, 90% H_2O and 10% D_2O) with the ratio of q = [DMPC]/[DHPC] = 0.5 under the inert nitrogen gas environment. This lipid suspension was allowed to hydrate at room temperature with vortexing and then subjected to heating (38 °C) and cooling (4 °C) cycles. Each cycle was followed by vigorous vortexing for 30 min at room temperature to ensure complete mixing of the lipids. These processes were carefully performed several times until the bicelle solution was cleared, which was taken after centrifugation. SP in buffer solution was then added drop-by-drop to the bicelle solution with vortexing until the molar ratio of peptide to lipids reached 1 : 40. The final concentration of SP in the bicelle solution was 2.8 mM, and the total amount of lipids was 15% (wt/wt). A minute amount of hexamethyldisilane (HMDS) was added to the bicelle solution to monitor the diffusion of the whole bicelle during the diffusion measurement [27].

NMR Spectroscopy

All NMR experiments were carried out on a Bruker DRX 500 Spectrometer (500.13 MHz for ^{1}H frequency) equipped with a broad-band inverse probe and pulsed field *z*-gradient capability. Two-dimensional TOCSY and NOESY were performed to obtain the spectral and sequential assignments of the peptides. Phase-sensitive NOESY and TOCSY spectra were collected with 256 scans, a relaxation delay of 2 s, a spectral width of 5000 Hz, and 4 K and 512 complex data points in t_2 and t_1 dimension, respectively. The NOESY spectrum was recorded with a mixing time of 200 ms. Temperature-dependent experiments were performed in the range of 295–325 K by an increment of 5 K. The stability of temperature was maintained within ±0.1 K during the experiments, and the sample was allowed to equilibrate for at least 1 h between the temperature changes.

Diffusion Measurements by PFG NMR

Double-stimulated-echo (DSTE) pulse sequence with bipolar gradient pulses was used for the diffusion measurements by PFG NMR, which has been shown to effectively suppress the convection artifacts [28]. In the DSTE experiment, the gradient-dependent signal attenuation f_G is described by the following equation:

$$f_G = \exp\left[-Dq^2\left(T + \frac{4\delta}{3} + \frac{5\tau_1}{4} + \frac{\tau_2}{4}\right)\right] \quad (1)$$

where D is diffusion coefficient, T diffusion time, δ gradient duration, and τ_i the settling time. The q is defined as $q = \gamma\delta g$, where γ is the magnetogyric ratio of nucleus and g the gradient strength. To avoid the relaxation effects in diffusion measurement, the gradient strength was varied with the diffusion time being constant. The diffusion times of 400–800 ms were used for the diffusion measurements of SP and bicelles, and 40–80 ms for water. The gradient duration was 2 ms, and the settling time was set to 30 ms. Sine-shaped spoiler gradient with 6.6 G/cm was applied for 4 ms in each *z*-storage period. Scans of 32 and 256 with phase cycling were obtained for the diffusion measurements of lipid and peptide signals, respectively. The gradient strength in a series of experiments was increased from 0.5 to 25 G/cm in ten steps. The diffusion coefficient was determined from the decay of PFG echo signals through a nonlinear least-squares fit of Eqn (1).

Determination of Peptide Partition from Diffusion Measurements

The partition coefficients of SP in the bicelle solution can be determined from the diffusion coefficients obtained by PFG NMR. The method for an analysis of the diffusion data was basically the same as that used for the determination of partition coefficients of SP in DPC micelles [4], which was based on the two-site model [29]. The decay of PFG echo signals can be approximated to a single-exponential when the exchange between the peptides in free and bicelle-bound forms is fast compared with the chemical shift differences between the two forms. Thus the measured diffusion coefficient D_{obs} obtained from the decay of PFG echo signals can be given by

$$D_{obs} = f_b D_b + (1 - f_b) D_f \quad (2)$$

where D_b and D_f denote the diffusion coefficients of the peptide in the bound and free forms, respectively, and f_b is the fraction of the bound peptides [4]. D_b can be taken as equal to the diffusion coefficient of the bicelles $D_{bicelle}$, which can be directly measured from the decay of resonances for lipids in the PFG NMR spectra. Alternatively, $D_{bicelle}$ can be measured from the signals of a small amount of hydrophobic molecule that is completely solubilized within the hydrophobic core of lipid bilayers. In the present study, HMDS was used as a probe molecule for the measurements of $D_{bicelle}$, and the $D_{bicelle}$ measured from HMDS was similar to the directly measured $D_{bicelle}$ of lipids within the experimental errors. D_f was obtained from the following equation by the measurements of D^b_{water}, D^0_f, and D^0_{water} [30]:

$$D_f = D^0_f \times (D^b_{water}/D^0_{water}) \quad (3)$$

where D^0_f and D^0_{water} are the diffusion coefficients of free peptide and water in buffer solution, respectively, and D^b_{water} is the diffusion coefficient of water in bicelle solution. The diffusion constants for water are introduced to account for differences in viscosity between the buffer and bicelle solutions. From the calculation of f_b derived from the measured diffusion coefficients with Eqn (2), the partition coefficient of the peptides in bicelles, p, defined as

$$p = [P]_{bicelle}/[P]_{aqueous} \quad (4)$$

where [P] is the peptide concentration which in the respective phase can be determined [30]. The peptide concentrations [P] are related to f_b by the following equations:

$$[P]_{bicelle} = n_p \times f_b / V_{bicelle} \quad (5)$$

$$[P]_{aqueous} = n_p \times (1 - f_b)/V_{aqueous} \quad (6)$$

where n_p is the total number of moles of peptide in the sample, $V_{bicelle}$ and $V_{aqueous}$ are the phase volumes of the bicelles and the

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aqueous bulk, respectively, and their ratio is approximated by their weight fractions [4].

Thermodynamic Functions from Temperature-dependent Diffusion Measurements

The thermodynamic function of partition was calculated from the temperature-dependent partition data. The Gibbs free energy of partition ΔG is related to the partition coefficient (equilibrium constant) by the following equation:

$$\Delta G = -RT\ln(p) \quad (7)$$

When the variation of the partition coefficient ln (p) with the inverse of temperature $1/T$ is close to being linear, the enthalpy and entropy changes (ΔH and ΔS) can be obtained from the slope and intercept of the plot of van't Hoff equation, respectively, assuming that ΔH is constant over the temperature range studied. On the other hand, if the temperature dependence of ln (p) is nonlinear, the thermodynamic functions ΔH°, ΔS° and ΔC_p can be determined from a nonlinear least-squares fit of the following equation by assuming a constant ΔC_p in the temperature range studied [4,6,31]:

$$\Delta G = \Delta H^\circ + \Delta C_p(T_{obs} - T_{ref}) - T_{obs}\Delta S^\circ - T_{obs}\Delta C_p \ln(T_{obs}/T_{ref}) \quad (8)$$

where the T_{obs} and T_{ref} are the observed and standard state reference temperatures, respectively.

Results

Chemical Shift Assignments

^{1}H NMR chemical shift assignments for SP in isotropic ($q = 0.5$) bicelles were performed by using the well-established procedure developed by Wüthrich [32]. TOCSY was primarily used to identify the spin systems of the residues, and NOESY was used to establish the sequential assignments. The absence of amide protons in Pro^2 and Pro^4 makes it difficult to assign the ^{1}H resonances belonging to two Pro residues, since the peptide was dissolved in nondeuterated bicelle solution, resulting in severe overlaps of the peptide and lipid resonances in the aliphatic region. All the assigned ^{1}H chemical shifts are summarized in Table 1.

Table 1. Summary of ^{1}H chemical shift assignments (in ppm) for 2.8 mM substance P in isotropic ($q = 0.5$) bicelles

Residue	NH	C_αH	C_βH	C_γH	Others
Arg^1	n/a	n/a	n/a	n/a	n/a
Pro^2	–	n/a	n/a	n/a	n/a
Lys^3	8.51	n/a	1.83, 1.97	1.62	C_δH 1.64
Pro^4	–	4.43	n/a	n/a	n/a
Gln^5	8.60	4.29	2.07	2.46, 2.36	$N_\delta H_2$ 6.92, 7.63
Gln^6	8.23	4.38	2.05	2.32	$N_\delta H_2$ 6.90, 7.52
Phe^7	8.40	4.48	3.05, 3.13	–	2,6H 7.22 3,5H 7.24
Phe^8	8.21	4.53	3.13, 3.38	–	2,6H 7.33 3,5H 7.35
Gly^9	8.08	4.02	–	–	–
Leu^{10}	7.99	4.40	1.71, 1.87	1.60	C_δH 1.03
Met^{11}	8.05	4.53	2.13, 2.24	2.58, 2.66	NH_2 7.13, 7.42

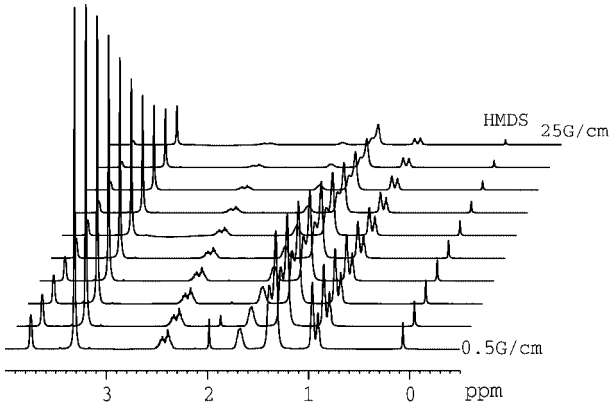

Figure 1. The decay of the signals of lipids and HMDS in isotropic ($q = 0.5$) bicelles at 300 K with an increment of the gradient strength in a series of ^{1}H PFG NMR experiments. The resonance of HMDS at ~0 ppm was used to measure the diffusion coefficients of bicelle-bound SP.

Partition of SP in Bicelles

Figure 1 shows the representative ^{1}H PFG NMR spectrum, where the signals of lipids (DMPC and DHPC) and HMDS (~0 ppm) in isotropic bicelles are decaying with single-exponential as the gradient strength is increased in a series of experiments. The gradient strength was increased from 0.5 to 25 G/cm in ten steps during the experiment, while the duration of gradient pulses and the diffusion time were kept constant. The resonance of HMDS was used to measure the diffusion coefficients of the bound SP (D_b). The measured D_b was also used to estimate the effective hydrodynamic radius of the bicelle [29], which was found to be about 3.7 nm over the temperature range studied. From the measured hydrodynamic radius of 3.7 nm, the diameter of the flat DMPC surface can be estimated to be 6 nm by taking the thickness of 4 nm for the DMPC bilayers, which is comparable with the reported value of 8 nm for the $q = 0.5$ bicelles [33,34]. In addition, the diffusion coefficients of the bicelles (D_b) were about the same before and after the addition of SP to the bicelle solution, indicating that the structure of bicelles was not disrupted by the binding of SP. Previous studies of several peptides in bicelle solutions have also reached to the same conclusion [10,20], where the size changes induced by the addition of peptides to bicelles were so negligible that they could not cause any changes or disruptions of the bicelle structures. Furthermore, if the exchange between the lipid monomer and the bicelles occurs significantly, the apparent diffusion coefficient obtained directly from the lipid signals should be higher than the value determined from the diffusion of the probe molecule, however, the decay rates of the lipid and HMDS signals in Figure 1 are basically the same, suggesting that the monomer–bicelle exchange is minimal maintaining the bicelles in good shape.

The decay of PFG echo signals of SP in the bicelle solution was fitted well with single-exponential, indicating that the assumption of fast exchange between two sites was valid. The diffusion coefficients of free SP (D_f) and a weighted average of free and bound SP (D_{obs}) were obtained from the well-separated aromatic resonances of Phe^7 and Phe^8 residues around 7.2 ppm. The partition coefficients (p) of SP and the fraction (f_b) of the bicelle-bound SP were evaluated from the measured diffusion coefficients at various temperatures, and are listed in Table 2 together with the diffusion coefficients D_b, D_f, and D_{obs}. The temperature dependence of the diffusion coefficients follows the

Table 2. The diffusion coefficients (in 10^{-10} m^2/s) and the partition of substance P in isotropic ($q = 0.5$) bicelles

	Temperature (K)						
	295	300	305	310	315	320	325
D_b	0.394	0.468	0.541	0.623	0.711	0.800	0.905
D_f	2.195	2.696	2.954	3.191	3.785	4.090	4.398
D_{obs}	0.634	0.756	0.896	1.040	1.235	1.425	1.688
f_b	0.882	0.871	0.853	0.840	0.824	0.810	0.779
P	108.9	98.5	84.7	76.6	68.2	62.3	51.5

Arrhenius relationship in all cases, indicating that the convection is effectively removed in the diffusion measurements by DSTE pulse sequence [28]. As can be seen in Table 2, the partition coefficient of SP decreases as the temperature is increased, indicating a favorable (negative) enthalpy change upon partitioning of the peptides.

Thermodynamic Functions for the Partitioning of SP

The Gibbs free energies for the partitioning of SP in isotropic bicelles were determined from the partition coefficients at various temperatures. Figure 2 shows the variation of the partition coefficient with temperature, where the slope of the ln (p) versus $1/T$ plot is close to being linear, indicating that the thermodynamics functions can be obtained from the van't Hoff equation with the assumption of relatively constant ΔH over the temperature range examined. Nevertheless, the thermodynamic functions for the partitioning of SP were determined at 300 K from the nonlinear least-squares fit between ΔG and T using the Eqn (8) and summarized in Table 3 together with the values for the partitioning of SP in DPC micelles. The remarkable features that can be seen in Table 3 are the negative entropy change (ΔS) as well as the negative enthalpy change (ΔH) in the partitioning of SP to bicelles. The large negative enthalpy change has been frequently observed in the partitioning of small peptides to micelles or other lipids [2,21]; however, the negative entropy change in bicelles is in sharp contrast with the positive entropy change in DPC micelles. In addition, the heat capacity change (ΔC_p), which could reflect the change in the strength of interactions in the bicelle system upon binding of SP, is significantly different from that in the DPC micelle system. The magnitude of ΔC_p is one order of magnitude larger than that in DPC micelles, while it is rather close to that of -150 cal K^{-1} mol^{-1} for lipid bilayers [3].

Discussion

The large negative enthalpy change of about -4 kcal mol^{-1} for the partitioning of SP in isotropic bicelles indicates that the partitioning is essentially enthalpy-driven process, which is generally interpreted as the nonclassical hydrophobic effect based on the van der Waals interactions between the nonpolar residues of solute and the hydrophobic core of the lipid bilayer [21]. This enthalpy-driven partition of SP was also observed in the binding of SP to the DPC micelles [4]. In fact, the large negative enthalpy change appears to be the general feature for partitioning of small amphipathic solutes and peptides to lipid bilayers. On the other hand, the negative entropy change for partitioning of SP in bicelles is in sharp contrast with the positive entropy change

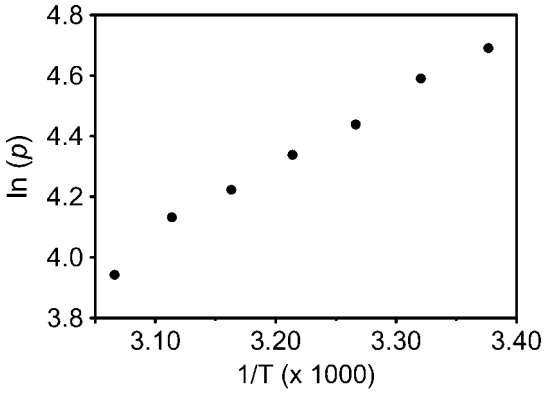

Figure 2. Temperature dependence of the partition coefficients for the partitioning of substance P in isotropic ($q = 0.5$) bicelles.

in DPC micelles. The positive entropy change for partitioning of peptides is considered to proceed mainly through the disordering of the deleted waters from the peptides and the hydrophobic acyl chains of the membrane lipids, while the negative entropy change could mainly result from the helix formation of the peptide upon its binding to lipid bilayers.

Previous studies on the partition of small peptides in POPC/POPG (3 : 1) vesicles revealed that the binding enthalpy of the peptide varied significantly with the vesicle size. Thus, the partitioning of the peptide was enthalpy-driven with a small negative entropy change of -4 cal K^{-1} mol^{-1} [21] or relatively large negative entropy change of -40 cal K^{-1} mol^{-1} [35] in SUV of 30 nm in diameter, while it was entropy-driven with a small positive enthalpy change (0.8 kcal mol^{-1}) in large unilamellar vesicle (LUV) with a diameter of 400 nm [21]. Such differences in the thermodynamics of partitioning between the SUV and LUV systems were explained by the differences in the degree of the packing of lipids. As the vesicle size increases, the curvature of the membrane surface decreases, resulting in relaxed packing constraint and enhanced internal tension of the lipids, which increases the entropy of the lipid matrix significantly upon peptide binding. Since the insertion of peptide into LUV requires more energy due to the internal tension than insertion into SUV, there is a large enthalpy–entropy compensation depending on the vesicle size, while the free energy of binding is almost independent of the vesicle size. Similar result of negative entropy change has also been observed for the peptide partition in neutral POPC vesicles, where the partitioning was enthalpy-driven with a negative entropy change (*ca* -20 cal K^{-1} mol^{-1}) in SUV of 20 nm in diameter [36]. However, the relative contributions of ΔH and ΔS to the ΔG in terms of the membrane curvature are completely different for the partitioning of SP in the micelle and bicelle systems (*ca* 3 and 8 nm in diameter), where the partitioning is enthalpy-driven with a negative entropy change of -4.3 cal K^{-1} mol^{-1} in bicelles of flat bilayer surface, while it is enthalpy-driven with a positive entropy change of 4.0 cal K^{-1} mol^{-1} in micelles of highly curved surface. Our result of enthalpy-driven process with relatively large negative entropy change is rather close to those in SUV vesicles than in DPC micelles [4]. Thus, the thermodynamics for the partitioning of amphipathic peptides is much more complicated than anticipated, and may depend on many factors including the peptide conformation, the types of lipids, and the charge distribution of membrane surface, though the curvature of membrane surface could still play an important role in the partitioning of peptides. Of course, there is a possibility that the SP binds to the curved

Table 3. Thermodynamic functions for the partitioning of substance P from water to lipids in the DPC micelle and isotropic bicelle systems at 300 K

	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal K^{-1} mol^{-1})	ΔC_p (cal K^{-1} mol^{-1})
Bicelles	-2.75 ± 0.10	-4.03 ± 0.19	-4.3 ± 0.6	-62.7 ± 44.1
DPC micelles[a]	-3.75 ± 0.08	-2.58 ± 0.10	4.0 ± 0.1	-5 ± 4

[a] Data for DPC micelles at 298 K were taken from Ref. 4.

surface of bicelles, since the area of the curved surface in the rim formed by DHPC is even larger than that of the flat surface formed by DMPC. However, the results of a number of previous studies [37–39] indicate that the peptides bind preferentially to the flat DMPC surface of bicelles presumably due to more compact packing of DMPC than DHPC in the curved rim. Even if we do not completely rule out the possibility of SP binding to the curved surface of bicelles, the contribution of SP binding to the flat surface should be still appreciable and could make a significant difference in the thermodynamics between the bicelle and micelle systems.

Heat capacity changes (ΔC_p) occurred in the partitioning of peptides from water to lipids could provide some information on the nature of molecular interactions in the lipid bilayer system. As can be seen in Eqn (8), ΔC_p determines how ΔH and ΔS respond to the temperature change of the system. The negative values of ΔC_p imply that some of the bonding or nonbonding interactions are disrupted upon peptide binding to lipid bilayers, which could be caused by the burial of hydrophobic side chains in the bicelles accompanying the release of water molecules from the hydrophobic core or surfaces [40]. It is noteworthy that the released water molecules may have lower heat capacity than the structured water molecules due to the disruption of interactions between the water and the membrane surface or hydrophobic core, resulting in the negative heat capacity change upon peptide binding. In addition, the contribution to the free energy change of partitioning from each hydrophobic side chain taking part in the interaction with the hydrophobic core was reported to be approximately -0.6 kcal mol^{-1} [41]. The measured ΔG value of -2.75 kcal mol^{-1} at 300 K for the partitioning of SP in bicelles is then consistent with a binding model [4,41] that consists mainly of the interactions of the hydrophobic side chains of Phe^{7}, Phe^{8}, Leu^{10}, and Met^{11} with the lipid matrix, though such conclusion on the binding mode of SP based on the thermodynamic functions of partitioning alone could be questionable.

In summary, we have measured the temperature dependence of the partition coefficient of SP in isotropic ($q = 0.5$) bicelles using PFG NMR diffusion technique. Thermodynamic analysis of the diffusion data shows that the partitioning of SP in bicelles is enthalpy-driven with relatively large negative entropy change, which is in sharp contrast with the partitioning of SP in DPC micelles, where the entropic term as well as the enthalpic term makes a favorable contribution (positive entropy change) to the partitioning of SP. The results thus suggest that the curvature of the membrane surface might play a significant role in the partitioning of peptides, though the partitioning process can be affected by many factors in a cooperative manner.

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